these substances was increased. c) Due to the specific allergic reaction of rats to Dextran, the relative effectiveness of this plasma expander could not be ascertained. 2. Hematocrit data recorded in terms of per cent of initial values are given for several animals in all groups. a) Hypertonic saline solutions tend to maintain a cell-plasma ratio more nearly approaching the initial than isotonic solutions. b) Increased hemodilution followed infusions of the higher concentrations of macromolecular substances. c) Hemodilution in general cannot be directly correlated with survival; however, the lowest hemodilution values were found, except for saline, in those groups having the lowest survival percentage.

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Cyclophorase System, XXII. Cyclophorase System of Rabbit Heart Muscle.* (19374)

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Previous studies in this laboratory have dealt with the reactions involved in the complete oxidation of pyruvic acid, fatty acids and certain amino acids by the cyclophorase-mitochondrial (CM)§ system of rabbit liver and kidney(1-6). The present report deals with some properties of the CM system of rabbit heart muscle. The heart system (cat) has been studied by Ochoa(7) with special reference to the oxidation of α-ketoglutarate, and (in the rat) by Lehninger(8) and Potter (9) with reference to fatty acid oxidation and oxidative phosphorylation, respectively. From the standpoint of systematics, it is important to know how the CM system of heart muscle compares with those of kidney and liver with respect to properties and metabolic spectrum. The present report deals with some comparative studies along these lines.

Preparation of the CM system. The heart (obtained immediately upon sacrifice of the animal) is divested of its pericardial sac and auricular tissue, and packed in ice (average weight, 5 g). The iced, ventricular tissue is cut into small pieces, added directly to 75 ml of 0.9% potassium chloride (0°C) in the small, monel metal, Waring blendor and blendorized for one minute. The resultant suspension is passed through a double layer of gauze to remove fibrous tissue and then centrifuged at 3-5°C for 7 minutes at 2500 x g. After discarding the supernatant fluid, the residue (R_1H) is resuspended in cold 0.9% potassium chloride and centrifuged again for 5 minutes. The residue (R_2H) is now made up to 6 ml with 0.9% potassium chloride. The Qo_2¶ of these preparations range from 33-45. The R_2H accounts for about 40% of the oxidative activity of the original homogenate. It was found in some but not all cases that when the R_2H is supplemented with the supernatant of the first centrifugation, the original oxidative activity can be reached. Phase microscopy of the preparation disclosed nuclei and nuclear fragments in addition to mito-

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§ For the association of cyclophorase activity with mitochondria *cf.* Harmon, *J. Exp. Cell Res.*, 1950, v1, 382.

¶ μl oxygen absorbed/hr/mg dry wt of preparation in presence of α-ketoglutarate at 38°C.

TABLE I. Effect of Cofactors.

	μl O_2 30 min	μl O_2 120 min
A. Complete system	1034	
no phosphate	357	
no adenosine-5-phosphate	129	
no substrate	0	
B. Complete system	650	1300
+ diphosphopyridine nucleotide (500 γ)	602	1250
+ cocarboxylase (200 γ)	600	1320
+ flavindinucleotide (60 γ)	580	1280
+ cytochrome c (350 γ)	650	1300

The complete system contained 1 ml of enzyme preparation, .2 ml of .1 M phosphate buffer, pH 7.4, .3 ml of .01 M adenosine-5-phosphate, .2 ml of .1 M $MgCl_2$ and 20 μ M α-ketoglutarate. Final volume 3 ml 38°C, oxygen in gas phase.

TABLE II. Specificity of the Adenine Nucleotide.

	μl O_2 80 min
System without adenine nucleotide	160
+ .3 ml .01 M adenosine-5-phosphate	1260
+ .2 ml .025 M adenosinediphosphate	1335
+ .1 ml .05 M adenosinetriphosphate	1333

The complete system contained 1 ml enzyme preparation, .2 ml of .1 M phosphate buffer, pH 7.4, .2 ml of .1 M $MgCl_2$ and 20 μM α-ketoglutarate.

chondria. A few intact myofibrils were also recognizable.

Methods. In most experiments the substrate was mixed with the enzyme system before the manometers were placed in the bath. This was necessary, since in absence of substrate, the enzyme system deteriorates rapidly. After a 5-minute equilibration period, the taps were closed. The oxygen uptake was corrected to include the equilibration period on the basis of the rate recorded during the first 5-minute period. The experiments on oxidative phosphorylation were carried out at 25°C. Inorganic phosphate was determined by both the Fiske-Subbarow(10) and Lowry-Lopez methods(11) with excellent agreement between the two methods.

Results. Components for maximum activity. Only 3 components are essential for maximum activity, *viz.* inorganic phosphate, magnesium ions and adenosine-5-phosphate (Table I, A). These requirements were shown previously by Ochoa(7) to apply to the CM system of cat heart muscle; in our own laboratory the same requirements were shown to apply to the CM system of rabbit liver and kidney. The addition of cytochrome *c*, diphosphopyridinenucleotide, cocarboxylase or flavinadenine dinucleotide had no effect either on the initial rate of oxidation or on the rate of decline of activity (Table I, B). The CM system of rabbit heart muscle as prepared above, shows a rapid decline in activity after the first 30 minutes. By contrast, the CM systems of rabbit liver and kidney show linear rates of oxidation for 1-2 hours. Adenosinemonophosphate can be replaced by either adenosinediphosphate or adenosinetriphosphate (Table II). The optimum levels of adenosine-5-phosphate and inorganic phosphate are the same in the heart system as for other systems. However, the optimum level of magnesium ions is about 5 times that described for the liver and kidney system. This requirement for higher magnesium levels is clearly demonstrable in a well-washed enzyme preparation (Table III), or in an aged enzyme preparation (Fig. 1). The addition of magnesium chloride to a fresh preparation (R_2H) produces little stimulation—whereas a preparation which has been aged for 4 hours at 0°C and then washed with saline solution, shows the full original activity only when supplemented with the higher level of magnesium chloride.

Metabolic spectrum. The diagnostic which we have used for an intact CM system is the ability to carry out the full citric acid cycle. The data of Table IV establish that the CM system of rabbit heart muscle catalyzes the complete oxidation of each of the intermediates of the citric acid cycle. With respect to

TABLE III. Magnesium Requirements.

	μl O_2 30 min
I. R_2H—no $MgCl_2$	452
+ .2 ml of .1 M $MgCl_2$	515
II. R_3H—no $MgCl_2$	164
+ .2 ml of .1 M $MgCl_2$	505
III. R_3H (centrifugation carried out with .9% KCl .0013 M $MgCl_2$)	522

The complete system contained 1.5 ml enzyme preparation, .2 ml .1 M phosphate buffer, pH 7.4, .3 ml .01 M adenosine-5-phosphate and 20 μM α-ketoglutarate.

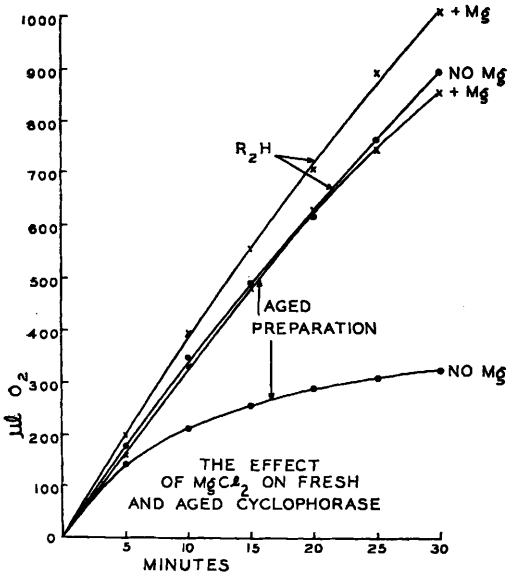

FIG. 1. Effect of magnesium ions in fresh and aged rabbit heart muscle CM preparations. The fresh preparation was brought to the stage of R_2H. The aged (4 hr) preparation was washed with .9% potassium chloride and brought to the R_3H stage. Details as in legend for Table III.

fatty acid oxidation,‖ the pattern is similar to that for the kidney system. According to manometric analyses (Table V), even-numbered fatty acids—including acetoacetate—are oxidized to carbon dioxide and water, whereas odd-numbered fatty acids are oxidized to carbon dioxide, water, and propionate. A well-washed enzyme preparation (R_3H) requires the presence of small amounts (1-2 μ moles) of some member of the citric acid cycle to initiate fatty acid oxidation. The oxidation of fatty acids is completely inhibited by 2:4-dinitrophenol at a level of 6.5×10^{-6} M (Table VI).

The oxidation of L-amino acids by the rabbit heart CM preparation has not yet been thoroughly investigated. L-glutamate is oxidized consistently at a rapid rate. Repeated attempts to demonstrate manometrically oxidation of lactate in the heart preparation were unsuccessful with or without supplements, such as diphosphopyridinenucleotide or the supernatant from the first centrifugation. Some lactic dehydrogenase activity could be detected by the anaerobic Thunberg technic.

Coupling of oxidation and phosphorylation. Ochoa(7), using cat heart muscle preparations, and Cross *et al.*(12), using rabbit liver and kidney CM preparations, have shown that esterification of inorganic phosphate accompanies all the oxidative steps of the citric acid cycle with an overall P/O ratio of about 3. Table VII shows a typical experiment with the CM system of rabbit heart muscle in which an average P/O ratio of 2.9 is attained.

Discussion. The CM system of rabbit heart muscle appears to share the basic properties of mitochondrial systems generally.

TABLE IV.
Oxidation of Citric Acid Cycle Substrates.

Substrate	µM added	Oxygen utilized, µatoms Observed	Theory
Pyruvate	16	80.7	80
α-Ketoglutarate	5	42.6	40
Succinate	5	34.8	35
l-Malate	5	29.7	30
Fumarate	5	32.6	30
Oxalacetate	3	14.9	15
Citrate	4	36	36

The complete system contained 1.5 ml enzyme preparation, .2 ml of .1 M phosphate buffer, pH 7.4, .3 ml of .01 M adenosine-5-phosphate, and .2 ml of .1 M $MgCl_2$.

TABLE V. Oxidation of Fatty Acids.

Substrate	µM	Oxygen utilized, µatoms Observed	Theory
Acetate	10	41.6	40*
Butyrate	10	115	100*
DL-β-hydroxybutyrate	30	151	135*†
DL-β-hydroxycaproate	10	94	75*†
Acetoacetate	9	70.5	72*
n-caproate	4	64.5	64*
Octanoate	2	43.7	44*
Propionate	20	0	140*
Acrylate	6	0	36*
n-valerate	5	33	30‡
Heptylate	2.5	35.4	30‡
Pelargonate	3.5	59.1	63‡
Δ 10-11 undecylenate	2	41.2	46‡

* Calculated for oxidation to CO_2 and H_2O.

† Theory for oxidation of only one isomer. For complete system, see Table IV.

‡ Calculated for oxidation to CO_2 and H_2O and propionate.

‖ The demonstration of fatty acid oxidation in CM systems prepared from pig heart, beef heart and pigeon breast muscle has not been as unequivocal as in the case of the CM system of rabbit heart. It has yet to be decided whether the CM systems of cardiac tissue generally can oxidize fatty acids.

TABLE VI. Effect of Dinitrophenol on Fatty Acid Oxidation.

	μatoms O_2 60 min
Complete system	
+ 3 μM succinate	8.3
+ " " + 5 μM caproate	79.9
+ " " + " " + 6.5×10^{-6} M 2,4-dinitrophenol	8.3

Complete system as in Table IV.

TABLE VII.
Coupling of Oxidation and Phosphorylation.

Time, min	Inorganic phosphate utilized, μM	O_2 uptake, μatoms	P/O
0- 5	12.9	4.3	3
5-10	12.2	4.3	2.8
10-15	12.2	4.3	2.8

The complete system contained 1 ml enzyme preparation, .2 ml .1 M $MgCl_2$, .3 ml of .01 adenosine-5-phosphate, .4 ml of .1 M phosphate buffer, pH 7.4, .1 ml of .3 M NaF, .1 ml of M glucose, .05 ml of yeast hexokinase and 30 μM pyruvate. 25°C.

The higher level of magnesium requirement is the only unusual feature of the heart preparation. As prepared, the system contains the full complement of enzymes and coenzymes necessary to implement the complete oxidation of members of the citric acid cycle, fatty acid oxidation and oxidative phosphorylation. The rabbit heart system is not as stable under working conditions as the CM system of rabbit kidney. A marked decline in activity at 38°C is usually observed after some 30 minutes. The instability of the working system may be referable either to impurities in the preparation, or to the instability of the mitochondrial unit itself.

Summary. The preparation and some properties of the cyclophorase-mitochondrial system of rabbit muscle are described.

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Cyclophorase System XXIII. Correlation of Cyclophorase Activity and Mitochondrial Density in Striated Muscle.* (19375)

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Investigations in several laboratories(1-4) have established that mitochondria contain the complete enzymatic apparatus for implementing the reactions of the citric acid cycle and for generating oxidatively high energy phosphate bonds. The functional aspects of mitochondria are covered by the term "cyclophorase activity". Thus, some relation might be anticipated between the physiological function, and either the mitochondrial density or cyclophorase activity of that same tissue.

In this study, crude enzyme preparations were made from muscles of different physio-

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